

Review Article

The Immune Response to Acute Focal Cerebral Ischemia and Associated Post-stroke Immunodepression: A Focused Review

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ABSTRACT: It is currently well established that the immune system is activated in response to transient or focal cerebral ischemia. This acute immune activation occurs in response to damage, and injury, to components of the neurovascular unit and is mediated by the innate and adaptive arms of the immune response. The initial immune activation is rapid, occurs via the innate immune response and leads to inflammation. The inflammatory mediators produced during the innate immune response in turn lead to recruitment of inflammatory cells and the production of more inflammatory mediators that result in activation of the adaptive immune response. Under ideal conditions, this inflammation gives way to tissue repair and attempts at regeneration. However, for reasons that are just being understood, immunosuppression occurs following acute stroke leading to post-stroke immunodepression. This review focuses on the current state of knowledge regarding innate and adaptive immune activation in response to focal cerebral ischemia as well as the immunodepression that can occur following stroke. A better understanding of the intricate and complex events that take place following immune response activation, to acute cerebral ischemia, is imperative for the development of effective novel immunomodulatory therapies for the treatment of acute stroke.

Key words: focal cerebral ischemia, cerebral ischemia, immune response, innate immunity, adaptive immunity, stroke-induced immunodepression

According to recent statistics, stroke remains the 4th leading cause of death and a leading cause of disability in the United States [1]. More alarming, and more relatable, however, are statistics that equate this to one stroke occurring roughly every minute, and a stroke death taking place approximately every 4 minutes [2]. Unfortunately, older stroke survivors comprise the majority of those who experience moderate to severe disability from stroke [3], requiring significant care, and resulting in lost productivity with a combined cost of more than \$33 billion in 2008 alone [4]. More recent

projections suggest exponential increases to nearly 200 billion dollars in the annual costs of medical stroke-related costs in 2030 with the growth in per capita medical spending expected to increase the fastest for those over 65 years of age [5]. Since advanced age is one of the risk factors for stroke, the number of patients affected by this condition can only be expected to increase with the ageing of the U.S. population known as the “Baby boomer” generation [6]. However, in spite of these grim statistics, there is currently only one approved therapy for stroke which is utilized in approximately 5%

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of eligible stroke patients nationwide [7,8]. It is therefore paramount to understand the endogenous mechanisms that are activated in response to acute stroke, so that novel therapies that take advantage of this understanding can be developed for the treatment of this devastating condition. Perhaps evidence of the incomplete understanding of the intricacies of immune activation following stroke are the several unsuccessful attempts to translate preliminary knowledge regarding immune activation into therapies for stroke.

The immune response is an important endogenous mechanism that is activated in response to focal cerebral ischemia. While some aspects of immune activation following focal cerebral ischemia, such as cytokine production and inflammatory cell infiltration into ischemic brain tissue, have been known for years [9-11], some of the complexities of the molecular mechanisms responsible for this immune activation are now just being uncovered. This review provides an overview and synopsis of the current knowledge regarding key aspects of innate and adaptive immune activation during acute focal cerebral ischemia. It also explores the phenomenon known as post-stroke immunodepression that occurs following stroke and the molecular mechanisms thought to be responsible for this immune dysfunction.

DAMPs and Cerebral ischemia:

According to the Danger model of immunity, the immune system is activated not only in response to exogenous pathologic stimuli, but also in response to endogenous “danger” signals [12, 13]. During transient or permanent cessation of cerebral blood flow, all the structural and signaling elements in the neuronal, glial and vascular compartments of the neurovascular unit are affected [14]. As a result of this cellular stress, danger signals are thought to be released from the dead and dying cells that constitute the neurovascular unit. These danger signals are characterized by danger associated molecular patterns (DAMPs) that allow these molecules to be recognized by effectors of the innate immune response [15]. These DAMPs are analogous to pathogen associated molecular patterns (PAMPs) expressed on the surface of Gram negative bacteria which are recognized by Toll-like receptor (TLRs). Some potential “danger” signals that have been identified to date include: High mobility group box 1 (HMGB1), heat shock proteins, Adenosine triphosphate (ATP), S100B proteins, heparin sulfate, DNA, RNA, oxidized Low-density lipoprotein (LDL), β -amyloid, hyaluronan etc [16, 17]. These DAMPs are collectively referred to as “alarmins” because they induce chemotaxis and interact with receptors on antigen-presenting cells, such as dendritic

cells, thereby linking innate immune activation to activation of the adaptive immune response [18].

Of these alarmins, HMGB1, a ubiquitous nuclear protein, released from damaged cells, is one of the most well-studied DAMPs in cerebral ischemia. HMGB1 levels are increased in the serum of patients with acute cerebral ischemia [19-22] and may predict functional outcome at 1 year following ischemic stroke [23]. HMGB1 also rapidly disappears from the ischemic core in experimental murine stroke consistent with release of this DAMP from dead or dying cells during cerebral ischemia [24]. Furthermore, Toll-like receptors (TLRs) 2 and 4, as well as the Receptor for Advanced Glycation End products (RAGE) receptor, have been shown to mediate the inflammatory effects of HMGB1 [25]. Not surprisingly, however, in spite of the well-documented inflammatory effects of this DAMP, recent evidence shows that HMGB1 may also play a role in recovery and neuronal plasticity during the repair phase of cerebral ischemia [26]. In addition, hyaluronan, a component of the extracellular matrix and a regulator of angiogenesis, modulates innate immune responses, via TLRs 2 and 4, and is increased in ischemic brain tissue in rodent experimental stroke [27]. Hyaluronan levels are also increased in the serum of patients with acute stroke [28].

In contrast, other DAMPs, such as ATP, also released from damaged cells following cellular injury, lead to activation of a class of purinergic receptors [29] that subsequently result in activation of the innate immune response via inflammasome activation [30]. A two-signal model, involving TLR signaling and inflammasome activation, has been proposed to explain the expression of inflammatory mediators such as IL-1 β . In this model, the first step of innate immune response activation occurs via TLR signaling which leads to the expression of the immature pro-form of IL-1 β . The second step involves inflammasome activation, via ATP, among other possible endogenous ligands, which then leads to inflammasome activation and the processing of pro-IL-1 β to mature IL-1 β via activated caspase 1 [30].

Toll-like receptor signaling in cerebral ischemia:

Toll-like receptors are germ line-encoded pattern recognition receptors that have been evolutionarily conserved from plants to humans. TLRs were first identified in drosophila as important in the innate immune response to infection [31]. However, it is now well-established that TLRs also play an important role in ischemia/reperfusion in many organs including the brain [32, 33].

Toll-like receptors are transmembrane proteins that contain a cytoplasmic toll/interleukin 1 (TIR) domain that transmit extracellular signals downstream.

Downstream TLR signaling occurs mainly via the MyD88 and TRIF signaling pathways [34]. Several TLRs have been shown to be expressed on neurons and TLRs 2 and 4 are up regulated in these cells in response to energy deprivation *in vitro* [35]. With regards to upstream receptor signaling, several TLRs have been shown to play an important role in the pathogenesis of acute cerebral ischemia [35-39]. In addition, gene expression studies carried out on human peripheral blood monocytes have implicated the TLR signaling pathway as one of the significantly differentially expressed canonical pathways in patients with acute ischemic stroke [40]. Furthermore, studies of human stroke have shown that increased expression of TLR4, on peripheral blood monocytes, in patients with acute ischemic stroke, correlate with stroke severity [41].

With regards to downstream TLR signaling, however, studies from our group employing neuronal cultures, in models of *in vitro* ischemia and oxygen glucose deprivation, and *in vivo* models of focal and global, using mice with disruption of MyD88 and TRIF signaling, showed that disruption of these downstream adaptors is not protective in cerebral ischemia [42]. Other groups also found that disruption of MyD88 and TRIF is not protective in different models of experimental murine ischemia [41, 43-45]. These results suggest that the downstream TLR signaling adaptors that mediate signaling downstream from the TLRs, already identified as important in cerebral ischemia, remain to be fully characterized or that these downstream adaptors, MyD88 and TRIF, may have other roles in the acute innate immune response to focal cerebral ischemia [46].

In addition to transmembrane pattern recognition receptors such as TLRs, intracellular pattern recognition receptors can detect cytoplasmic DAMPs and activate the innate immune response. One such class of innate immune receptors are the NOD-like receptors (NLRs) which contain NACHT, PYRIN, leucine-rich repeat (LRR) and Caspase activation and recruitment domains (CARDs) domains and detect, among other ligands, ATP leading to inflammasome activation [47]. Specifically, NOD-like receptor family, pyrin domain containing 1 gene (NLRP1), which is predominantly expressed in the brain, has been implicated in focal cerebral ischemia [48]. In addition, NLRP3/NALP3 which is expressed both peripherally and centrally, is involved in the pathogenesis of inflammatory syndromes such as Muckle-Wells syndrome which includes stroke as part of the clinical disorder [49]. NLRP3/NALP3 has also been recently shown to mediate inflammatory responses in the brain in a model of experimental murine ischemia [50]. Other cytoplasmic innate receptors are primarily involved in responses to infectious stimuli and not in the immune response to acute focal cerebral ischemia. These

include RIG-I-like receptors (RLRs) which recognize intracellular viral RNA and AIM2-like receptors (ALRs), which include Absent in Melanoma-2 (AIM-2) and Interferon gamma-inducible protein 16 (IFI16) which recognize intracellular bacterial DNA [17].

Inflammatory mediators and focal cerebral ischemia:

Cytokines and chemokines

The expression of inflammatory mediators occurs both centrally and peripherally following focal cerebral ischemia. However, most of the evidence for central expression of inflammatory mediators has come from experimental stroke models and that for peripheral expression of cytokines and chemokines from studies of human stroke. Several cytokines and chemokines are expressed centrally in experimental stroke [51]. Some of these cytokines and chemokines, such as IL-6 [52, 53], IL-1, [54], [55-57], Tumor necrosis factor- α (TNF- α), [58] [57] [59] and Monocyte chemoattractant protein 1 (MCP-1) [60], are expressed in ischemic murine or rodent brain tissue. In human stroke, several studies have reported peripheral expression of a number of cytokines and chemokines, following focal cerebral ischemia, and serum levels of a number of inflammatory mediators such as IL-6 [61, 62] and TNF- α [63] [51] have been shown to correlate with infarct size and stroke severity. Peripheral cytokine and chemokine expression have also been demonstrated in experimental stroke [51, 64],[65].

The roles of the above cytokines and chemokines in cerebral ischemia have been explored in several knock-out, over expression, antibody neutralization or antagonization studies in experimental and human stroke studies [51]. Of these cytokines, IL-1 is one of the well-studied in cerebral ischemia. IL-1 is unique because receptors for both IL-1 and IL-18, are members of the IL-1 and Toll-like receptor super family [66]. As a result, both TLRs and IL-1 and IL-18 share a Toll-like receptor/Interleukin 1 Receptor transmembrane or TIR domain through which members of this superfamily transmit signals via downstream adaptors such as MyD88 into the cytoplasm [66].

Furthermore, antibody neutralization of IL-1 [67] or administration of IL-1 receptor antagonist [56, 68-72] is protective against experimental stroke. These promising results led to confirmation of safety and tolerability of IL-1 receptor antagonist in patients with acute stroke in a phase II clinical trial [73]. It remains to be seen whether this type of therapeutic strategy targeting the acute phase of inflammation, in human stroke, will be efficacious.

In contrast, cytokines such as IL6 and TNF- α appear to have biphasic or perhaps polyphasic roles in cerebral ischemia. For instance, deletion of IL-6 in experimental

stroke does not confer any protection against focal cerebral ischemia [53] or leads to increased infarct volume compared to wild type mice [74]. This lack of protection against cerebral ischemia by IL-6 occurs in spite of many studies, in experimental stroke and in humans, showing that levels of this cytokine correlates with infarct size, stroke severity and outcome from stroke [61, 63, 75]. These findings are similar to those obtained from our group in which mice with disruption of MyD88, a downstream TLR signaling adaptor, which mediates IL-6 expression [65], were not protected against focal cerebral ischemia [42]. Evidence for the dual role of IL-6 in cerebral ischemia is suggested by recent studies showing impaired neurogenesis in mice lacking IL-6 [76] probably secondary to the suggested role of this cytokine in the regulation of neural stem cell function [77].

Similar to IL-6, TNF α is also known to have a plurality of roles in cerebral ischemia [78] [79]. In experimental stroke for instance, TNF- α is known to be neuroprotective because it induces preconditioning to subsequent damaging ischemia [70, 80]. However, there is corresponding evidence for a neurotoxic role for this cytokine. Studies supporting a neurotoxic role for TNF- α include studies that show that chronic increased expression of TNF- α leads to increased susceptibility to focal cerebral ischemia [81]. Also, TNF- α receptor knockout mice are protected against cerebral ischemia [82]. Since chronic over expression or chronic deprivation, via receptor knockout, of this inflammatory cytokine does not recapitulate what occurs endogenously under normal conditions, earlier studies sought to determine the effect of acute inhibition of TNF- α on focal cerebral ischemia. Such studies which involved acutely antagonizing the effects of TNF- α were neuroprotective against experimental stroke [83, 84].

The overall conclusion from these studies is that several cytokines such as IL-1, IL-6 and TNF- α , among several others, are expressed both centrally and peripherally in response to brain ischemic injury, in acute stroke, and some of these cytokines play divergent roles during different phases of the injury response. The next phase of research into the roles of these incredibly complex proteins, during acute stroke, will have to lead to understanding of the upstream pathways that regulate expression of these cytokines and understanding the downstream pathways that these cytokines modulate as well as the pathways that these cytokines feedback upon to amplify inflammation. This type of knowledge is beginning to emerge with studies showing that cytokines such as IL-6 also regulate neural stem cell expression and neurogenesis [76,77]. In addition, studies showing that certain alarmins, such as periredoxin, can amplify the inflammatory response during cerebral ischemia [85]

are a step in the right direction in further dissecting the molecular basis of the complexities of inflammatory amplification during cerebral ischemia and the potential roles of specific cytokines in this process.

Adhesion molecules

Activation of the innate immune system in response to acute focal cerebral ischemia leads to cytokine expression and up regulation of adhesion molecule expression. The vascular endothelium can be activated by a number of inflammatory stimuli. These include cytokines such as IL-1, TNF- α , Interferon-gamma (IFN- γ) or underlying vascular pathogenesis, such as hypertension [86] or focal cerebral ischemia. These inflammatory stimuli ultimately leads to the up regulation and expression of leukocyte adhesion molecules capable of recruiting large numbers of leukocytes to the site of tissue ischemia. The leukocyte adhesion molecules that are up regulated on the surface of vascular endothelium following acute focal ischemia can be broadly classified into selectins, integrins and the immunoglobulin superfamily [87]. Up regulation of these cellular adhesion molecules permit cell-cell interactions as well as cell-extracellular matrix interactions which results in rolling and transmigration of immune cells from the periphery, via the endothelial basement membrane, into the brain parenchyma following cerebral ischemia.

Selectins:

The selectins are type 1 transmembrane glycoproteins that mediate the initial recruitment, homing, margination and “rolling” or low-affinity transient binding of leukocytes to the capillary endothelium during focal cerebral ischemia. The selectins consist of 1) P-selectin which is expressed on the surface of both platelets and endothelium, 2) E-selectin which is expressed only on activated endothelium and 3) L-selectin which is expressed on both endothelium and leukocytes.

In endothelial cells, P-selectin is stored in Weibel-Palade bodies [88] and in resting platelets is stored in alpha granules [89]. Since P-selectin is stored in the cytoplasm, it is released and expressed on the cell surface very rapidly during experimental focal cerebral ischemia [90]. In experimental stroke, mice with deficiency of P-selectin are protected against focal cerebral ischemia and have decreased polymorphonuclear leukocyte accumulation and decreased mortality following stroke [91,92]. Acute functional inhibition of P-selectin with monoclonal antibodies, in wild type mice, following experimental focal cerebral ischemia, also resulted in decreased infarct

volume and decreased evidence of “no-reflow” phenomenon following reperfusion [91].

However, in models of experimental global ischemia, acute inhibition of P-selectin, via monoclonal antibody administration, resulted in decreased survival of treated animals [93]. These divergent results may be reflective of possible differences in the role of P-selectin in focal versus global ischemia, or differences in inhibiting P-selectin function, via monoclonal antibody administration, prior to, versus following, cerebral ischemia.

Studies have also shown that inhibition of selectin-mediated activities by sCR1sLex; a soluble form of complement receptor 1, modified by sialyl Lewis X carbohydrate side chains, which has been shown to block P-selectin-mediated binding in vitro [94], resulted in decreased leukocyte and platelet recruitment and decreased infarct volume in experimental stroke [95]. In addition, there are reports that link P-selectin, and perhaps other selectin-mediated signaling events, to expression of adhesins that mediate firmer binding to the endothelium. More recently, P-selectin deficient mice have been shown to have decreased blood brain barrier disruption as assessed by gadolinium extravasation on brain MRI, as well as decreased leukocyte recruitment, though infarct size was not decreased in P-selectin knock-out mice in this study [96] as previously reported [91], thereby calling into question the strength of these findings. In addition, in non-human primates, humanized E/P selectin antibodies were protective against experimental stroke [97]. Lastly, in human stroke, increased P-selectin expression correlates with stroke severity [98].

E-selectin is unique because it is only expressed on activated endothelium. However, in contrast to P-selectin which is stored within granules in the cytoplasm of endothelial cells and platelets, E-selectin is transcribed de novo. Hence, it is expressed in sequence after P-selectin during focal cerebral ischemia. E-selectin binds to the sialyl Lewis^x and Lewis^A containing receptors on leukocytes. A number of studies in experimental stroke have shown up regulation of E-selectin expression on activated endothelium within few to several hours following focal cerebral ischemia. However, in human stroke E-selectin levels were not elevated in the serum [99]. In spite of these findings, transnasal E-selectin tolerization has been shown to induce tolerance to subsequent damaging cerebral ischemia in experimental stroke [100] and studies are currently underway to advance these studies to clinical trials with the ultimate goal of using this form of immunomodulation for the secondary prevention of stroke.

L-selectin, which is expressed on both endothelium and leukocytes, was originally identified as important in

homing to the sites of infection and to peripheral lymph nodes [101-103]. Subsequently, L-selectin has been shown to play a role in neutrophil margination and rolling along the activated endothelium [104]. However, acute inhibition of L-selectin, using monoclonal antibodies, did not confer protection against experimental stroke in rabbits [105]. Furthermore, in the few studies that have examined the expression of this adhesion molecule in humans, L-selectin levels have not been found to be elevated in patients with acute stroke [106].

Immunoglobulin super family:

Members of the immunoglobulin superfamily mediate stronger binding of leukocytes to the vascular endothelium than the selectins, during focal cerebral ischemia. The 5 members of the immunoglobulin superfamily include Intercellular adhesion molecule-1 (ICAM-1), Intercellular adhesion molecule-2 (ICAM-2), Vascular cell adhesion molecule-1 (VCAM-1), Platelet-endothelial cell adhesion molecule-1 (PECAM-1) and Mucosal vascular addressin cell adhesion molecule 1.

Of these, ICAM-1 is one of the well-studied in cerebral ischemia. In experimental stroke, for instance, ICAM-1 is constitutively expressed on endothelium, but is up regulated in response to specific cytokines, following focal cerebral ischemia and binds to lymphocyte function associated antigen-1 (LFA-1) present on polymorphonuclear leukocytes [87]. ICAM-1 deficiency [107], [108] or acute inhibition of ICAM-1 with anti-ICAM-1 antibodies [109] is protective in experimental stroke. In addition, in human stroke, levels of soluble ICAM-1 increase in serum acutely following stroke [110]. Post-mortem studies of patients, who died following stroke, also show increased ICAM-1 expression in ischemic brain tissue [111-113]. In spite of the above findings in experimental stroke, and in human stroke, suggesting an important role for ICAM-1 in cerebral ischemia, clinical trials in humans aimed at blocking ICAM-1 function, via monoclonal antibody administration, did not show any neuroprotection against cerebral ischemia [114]. The reasons for the failure of this type of immunomodulation in stroke patients are probably complex and may relate to the mode and timing of functional inhibition of leukocyte recruitment due to the incomplete understanding of the possible plurality of roles of these adhesion molecules in acute ischemic stroke in patients with multiple risk factors and other underlying comorbid conditions.

ICAM-2, on the other hand, though less well-studied, is expressed on both activated endothelium and platelets raising the possibility that this adhesion molecule may mediate platelet-endothelial cell

interactions [115]. The role of this adhesion molecule in focal cerebral ischemia remains to be established.

Lastly, there is contradictory evidence regarding the role of VCAM-1 in focal cerebral ischemia. The expression of VCAM-1, which binds to Very late antigen-4 (VLA-4), on polymorphonuclear leukocytes, is increased following focal cerebral ischemia. In addition, mice with deficiency of VCAM-1 have reduced infarct sizes compared to wild type mice [116]. However, acute inhibition of VCAM-1, using monoclonal antibodies, did not result in protection against experimental stroke in models of reperfusion [117]. These apparently inconsistent results could be due to the differences between the effects of chronic deficiency of this molecule versus acute functional inhibition during acute focal cerebral ischemia. Furthermore, differences in the inflammatory signaling cascade, and mediators produced following ischemic stroke with reperfusion, compared to ischemic stroke with no reperfusion, could explain, in part, these differences. Finally, very little is known about the role of PECAM-1 and other ICAM subtypes such as ICAM-3 in focal cerebral ischemia.

Integrins:

Integrins are transmembrane glycoproteins that mediate cell-extracellular matrix and cell-cell interactions. These glycoproteins consist of α and β subunits and mediate firm interactions or adherence of leukocytes to the endothelium. Leukocyte integrins have a common β -chain; CD18 and different α chains; 11a, 11b and 11c. [118]. The most well-studied members of the integrin family in cerebral ischemia are the β 2 integrins which include LFA-1 or (CD18/CD11a) which is expressed on the surface of all leukocytes and Mac-1 or (CD18/CD11b) which is expressed on neutrophils, monocytes and natural killer cells [118]. Mac-1 is stored in granules within the cytoplasm, of polymorphonuclear cells and monocytes, and can be transported to the cell surface rapidly following leukocyte activation. In contrast, LFA-1 is constitutively expressed and further up regulated following leukocyte activation [118]. A third member of the β 2-integrin family, p150, 95 or (CD18/CD11c) has not been well studied in cerebral ischemia.

LFA-1 mediates higher affinity binding of leukocytes to the vascular endothelium via endothelial adhesion molecules, ICAM-1 and ICAM-2, thereby resulting in firm adherence and arrest of leukocytes on the endothelial surface [118]. Evidence supporting the role of β 2-integrins in ischemic stroke immunopathogenesis comes from studies showing that chronic deficiency of LFA-1 or Mac-1 [119] or acute inhibition of Mac-1, via monoclonal antibody

administration, following cerebral ischemia [120], leads to reduction in infarct volumes and decreased polymorphonuclear cell accumulation in experimental stroke with reperfusion. In addition, membrane expression of CD11a and CD18 on peripheral leukocytes was up regulated in a small study with 10 patients with ischemic stroke and in 7 patients with transient ischemic attack [121].

Taken together, results of experimental, and limited studies of human stroke and TIA, suggest that adhesion molecules may play a more important role in focal cerebral ischemia with reperfusion compared to cerebral ischemia without reperfusion.

Leukocytes:

Several immune cells infiltrate the brain from the periphery following acute focal ischemia. Activation of resident immune cells also occurs locally following focal cerebral ischemia. Infiltration of leukocytes into the brain occurs following activation of the endothelium and increased expression of adhesion molecules on the surface of both endothelium and leukocytes. Most of the studies of leukocyte infiltration into the brain following cerebral ischemia have occurred in experimental stroke. In human stroke, studies of leukocyte involvement in stroke have been limited to evaluating levels of peripheral leukocytes or histological examination of post-mortem brain tissue. Recruitment of leukocytes to the brain occurs in a synchronized manner following focal cerebral ischemia. In this sequence, one of the first immune cells to “infiltrate” the ischemic brain are neutrophils, followed by monocytes, and then lymphocytes [122].

Neutrophils:

The first demonstration of early granulocyte infiltration into ischemic brain tissue occurred in studies showing accumulation of labeled granulocytes in ischemic brain tissue in the first few hours following focal cerebral ischemia [123]. Several studies have subsequently confirmed the findings of this initial study [124-126]. In addition, a phenomenon of microvasculature occlusion following cerebral ischemia referred to as “No-reflow”, first described in a model of rabbit experimental ischemia [127], has been subsequently attributed to polymorphonuclear cell occlusion of micro vessels in a model of non-human primate experimental stroke [128]. More recent studies have also shown that early neutrophil infiltration into ischemic brain tissue occurs in both models of transient and permanent ischemia, even though neutrophil infiltration occurs earlier when reperfusion is present compared to when there is no

reperfusion [129]. However, there is evidence to suggest that these neutrophils do not actually migrate across the glia limitans to “infiltrate” the ischemic brain parenchyma, but instead accumulate in the luminal surface of the perivascular component of cerebral microvessels [130]. Since vascular structures in the brain, such as the post-capillary venules, do not contain a true glia limitans [131] and since the glia limitans may be modified during the acute inflammation that accompanies focal cerebral ischemia, it is conceivable that some neutrophils might “infiltrate” the brain parenchyma during cerebral ischemia.

Regardless, in models of experimental stroke, the role of neutrophil accumulation in the brain following cerebral ischemia runs the gamut from neutrophils playing a detrimental role [132] [133, 134] to no clear involvement of neutrophils in stroke immunopathogenesis [135, 136]. In human stroke, brain neutrophil accumulation was noted within 24hrs of stroke onset using brain neutrophil imaging and confirmed in selected cases post-mortem, but stroke severity and outcome was not related to neutrophil accumulation [137]. Peripheral neutrophil levels were also elevated in patients with acute stroke without underlying infection [138]. Finally, in clinical trials of patients with stroke in which neutrophil accumulation, or adhesion of neutrophils to activated endothelium, was targeted therapeutically, no clear efficacy was shown in these studies [114, 139].

Taken together, these results showing differing effects of neutrophil depletion or inhibition on acute focal cerebral ischemia, suggest that the role of neutrophils in cerebral ischemia remains incompletely defined and needs to be further clarified using cutting edge molecular and imaging techniques. These types of evaluations should help to conclusively determine whether or not therapeutic approaches targeting this leukocyte can be successful in the treatment of human stroke.

Monocytes/macrophages:

Resident brain microglia, blood monocytes and tissue macrophages all play an important role in the innate immune response to focal cerebral ischemia [140]. In addition, both microglia and macrophages engage in phagocytosis of cells and cellular debris following focal cerebral ischemia, but there is evidence to suggest that microglia may play a more prominent role in phagocytosis compared to tissue macrophages or monocytes following focal cerebral ischemia [141, 142].

Microglia which are of hematopoietic origin [143], are normally maintained in a state of quiescence by local interactions between the microglia receptor CD200 and

its ligand, CD200R, on neighboring neurons [144, 145]. However, in response to focal cerebral ischemia, microglia are rapidly activated, as a result of the loss of the normal interactions with adjacent neurons, among other factors, and these activated microglia can be demonstrated in ischemic brain tissue within 24 hours following cerebral ischemia [146].

Blood borne monocytes are recruited from the periphery during focal cerebral ischemia and can be seen in the ischemic and peri-infarct brain tissue from 24 hours up to 14 days after experimental stroke [147] [146]. Human blood monocytes consist of classic proinflammatory CD14⁺ cells and reparative CD16⁺ monocytes [148]. The inflammatory monocytes give rise to M1 macrophages and are thought to be involved in the acute inflammatory response to cerebral ischemia, while reparative monocytes give rise to M2 macrophages and are thought to have an important role in tissue remodeling during the reparative phase of cerebral ischemia [149].

In contrast, brain macrophages are localized to the perivascular space between the endothelial basement membrane and the glia limitans [150]. Following focal cerebral ischemia, the number of cells in the perivascular cuff of cerebral microvessels that stain for macrophage cell surface markers increase significantly [60] and it is thought that these perivascular macrophages are continuously replenished by monocyte precursors in the blood [150-152].

The role of monocytes and macrophages in cerebral ischemia has also been inferred through the pattern of expression of chemokines such as monocyte chemoattractant protein-1 (MCP-1), since over expression of this chemokine results in increased mononuclear infiltrate into the brain [153]. In this regard, studies in experimental stroke show increased expression of MCP-1, message or protein levels, in the brains of animals subjected to either permanent or transient cerebral ischemia [154-157]. In addition, functional studies have shown that mice with deficiency of MCP-1 have reduced infarct volumes [158]. Conversely, mice over expressing MCP-1 have increased infarct volumes and increased infiltration of macrophages and monocytes into the brain [60]. More recent studies suggest that deficiency of MCP-1 specifically leads to decreased influx of blood mononuclear and polymorphonuclear cells into the ischemic brain [146]. In human stroke, monocyte levels are increased in the blood of stroke patients, but these monocytes are functionally deactivated [159,160]. Collectively, these studies suggest that monocytes/macrophages may play a detrimental role in the acute phase, but a reparative role in the chronic phase of focal cerebral ischemia.

Lymphocytes:

Lymphocytes are another class of leukocytes that contribute to the pathogenesis of stroke. Studies in experimental stroke have shown that lymphocytes accumulate at the site of ischemia, and in the border zone, as early as 24 hours, following cerebral ischemia [161]. Other studies in experimental stroke showed that recombination activating gene 1 (RAG1^{-/-}) knock-out mice, with no functional T and B cells, [162] are protected against experimental stroke. Furthermore, lymphocyte-deficient, Rag2^{-/-} mice have significantly smaller infarct volumes compared to their wild-type littermates [163,164]. However, reconstitution of peripheral lymphocytes in these mice results in significantly increased infarct volumes compared to wild-type mice [164].

Peak T lymphocyte accumulation, most of which are CD8⁺ cells, occurs around 7 days following cerebral ischemia [161]. Lymphocyte reconstitution studies further revealed that these CD8⁺ cells, more so than CD4⁺ cells, and not B cells, may contribute to the inflammation and thrombogenesis during cerebral ischemia [163]. In addition, consistent with a more significant role for CD8⁺ T cells in the immunopathogenesis of acute stroke are studies showing protection against experimental stroke with knock-out of perforin which mediates key cytotoxic effects of these T cells [164]. Finally, depletion of CD8⁺ T cells is more protective against experimental stroke compared to CD4⁺ T cell depletion [126].

The role of other T lymphocyte subsets, such as Tregs, have shown inconsistent results. On the one hand, in studies of immunological tolerance to experimental stroke, Tregs have been shown to be important in the development of immunological tolerance to experimental ischemia [165]. On the other hand, more recent studies with the DEREK mouse, in which Tregs can be acutely depleted with diphtheria toxin, show that acute depletion of Tregs is protective against cerebral ischemia and reconstitution or recovery of the native Treg replete state is not protective against experimental stroke with reperfusion [166]. In addition, lack of protection against experimental stroke was noted in scurfy mice which have immunologically defective, but phenotypically correct Tregs suggesting that the detrimental effect of Tregs in experimental ischemia is not due to their immunological function, but instead due to the presence of these cells [166].

Yet another T cell subset, the $\gamma\delta$ T cells, which are uniquely positioned between the innate and adaptive immune responses, plays a detrimental role in the pathogenesis of experimental stroke during the late phase, but not during the early phase of cerebral

ischemia [167]. In these studies, both blockade of T cell infiltration with the immunosuppressant, FTY720 and depletion of $\gamma\delta$ T cells reduced brain injury secondary to experimental stroke with reperfusion [167]. In other studies, depletion of these $\gamma\delta$ T cells conferred protection against cerebral ischemia in mice [168]. Production of IL-23 by infiltrating macrophages, on the other hand attracts $\gamma\delta$ T cells to the site of brain ischemia while production of IL-17 by these $\gamma\delta$ T cells was shown to mediate their inflammatory effects [167].

The role of lymphocytes in experimental ischemia is further suggested by studies showing protection against experimental stroke with reperfusion, when T cell infiltration is acutely blocked with antibodies to $\alpha 4$ integrin which mediates T cell binding to Vascular cell adhesion molecule 1 (VCAM-1) on the endothelium [169]. More recent studies, however, contradict these earlier studies by showing no protection against stroke in both transient and permanent experimental ischemia models when VLA4 is blocked with anti-CD49d monoclonal antibodies before cerebral ischemia [170].

Lastly, in experimental stroke, and also in human stroke, there is a profound lymphocytopenia that is thought to predispose patients to post-stroke immune dysfunction and infection [171] [172], but surviving T cells possess cell surface markers consistent with T-cell activation and have normal function [22].

In summary, the role of T cells during focal cerebral ischemia is illustrated by neuroprotection following antibody mediated complete lymphocyte or specific T cell subset depletion, as well as following inhibition of T cell binding to the endothelium. The temporal effects of T cell depletion and the prominent effects of T cell or lymphocyte depletion in models of reperfusion also suggest that lymphocytes play a role both in the early and late phases of focal cerebral ischemia and perhaps more in ischemia with reperfusion versus permanent ischemia.

Overall, these studies show that T cells play a significant role in stroke immunopathogenesis. Therefore, attempts to target T cell infiltration, post stroke, may have therapeutic potential, but it remains to be seen whether targeting lymphocyte infiltration will be of benefit in human stroke.

Complement activation and focal cerebral ischemia:

The complement system is a proteolytic enzymatic cascade with over 30 soluble and membrane-bound proteins that forms an important part of the innate immune response [173]. Components of the complement cascade are produced in the liver [174], but these components can enter the CNS through a disrupted blood brain barrier (BBB). However, *in situ* hybridization

studies have shown that complement factors are produced locally within the CNS by astrocytes, microglia, neurons and oligodendroglia [173, 175, 176].

The complement pathway is activated via 3 major pathways [177] and a more recently described 4th or extrinsic pathway [178, 179]. The 3 major pathways consist of the 1) The Classical pathway which is activated by C1q recognition of antibody and antigen immune complexes or IgM. 2) The Alternative pathway which is activated by binding of carbohydrate-containing antigens to mannose-binding lectins (MBL) and MBL-associated proteases; MASP1, MASP2 and MASP3 and Ficolins (M-Ficolin, L-Ficolin and H-Ficolin) and the 3) Alternative pathway which is activated via spontaneous hydrolysis of C3 in plasma. All 3 major complement activation pathways lead to activation of C3 via C3 convertase. The larger C3 cleavage product, C3b, opsonizes PAMPs and may also play a role in the clearance of DAMPs. C3b, along with other factors, can form the C5 convertase which activates C5, thereby amplifying the inflammatory cascade. In addition, C3b scavenges C3b-receptor expressing leukocytes. The association of C5b, C6, C7 and C8 result in the polymerization of C9 molecules which form the terminal transmembrane-pore forming membrane attack complex (MAC) [177].

Experimental stroke studies have also shown increased deposition of C3b in ipsilateral ischemic brain tissue compared to the non-ischemic contralateral hemisphere [180]. Studies of post-mortem brain tissue from patients with acute cerebral ischemia also show deposition of complement components C1q, C3C and C4d in ischemic lesions in contrast to control brains which do not show deposition of these products of activated complement [181]. In addition, stroke patients show decreased serum levels of MAC [182]. Similar to the results of human studies, ischemic brain tissue from experimental stroke models show increased levels of activated complement pathway factors in areas of ischemic brain tissue compared to the contralateral hemisphere [180]. Recent studies also show that acute stroke patients with single nucleotide polymorphisms that result in a MBL^{low} phenotype, and associated decreased levels of serum C3 and C4, have better 90-day outcomes following stroke [183]. In addition, mice with deficiency of MBL have significantly smaller infarct sized compared to their WT littermates [183].

The 4th or extrinsic pathway is activated by blood clotting factors, thrombin, fibrinolysis pathway products and other serine proteases which cleave complement components, C3 and C5, independent of their respective convertases [177]. Both C3a and C5a are anaphylatoxins that do not contribute to the formation of the MAC. However, C5a exerts chemotactic and potent

inflammatory effects. C3a in contrast mediates local inflammatory responses [177]. Of possible relevance to cerebral ischemia, patients with acute myocardial infarction treated with the serine protease, recombinant tissue plasminogen activator (tPA), showed peripheral complement activation as evidenced by increased serum levels of C3a and C5a [184] consistent with activation of the extrinsic pathway by fibrinolysis pathway products. Both C3a and C5a signal via the G-protein-coupled receptors, C3R and CD88 respectively. However, consistent with known biphasic roles of inflammatory mediators in the CNS, results from experimental mouse models show that CD88 is expressed during normal brain development and may have a role in neurogenesis and synaptogenesis [177].

Lastly, complement activation provides a bridge to the adaptive immune response by modulating different aspects of B and T cell function as well as survival.

Adaptive immunity

The adaptive immune response has been shown to be activated following acute cerebral ischemia with evidence for antibody production, T cell infiltration and differentiation in patients with acute stroke.

Cell-mediated immunity; T-cell mediated:

Some of the evidence for an adaptive immune response, with T cell involvement, in stroke comes from studies showing the protective effects of tolerance to CNS antigens in experimental stroke. In these studies, oral tolerance to CNS antigens, such as Myelin basic protein (MBP), was protective against experimental rodent focal ischemia [185]. This effect was thought to be mediated by regulatory T cell suppression of pathogenic Th1 immune responses. Subsequent studies confirmed this hypothesis and showed that the protection against focal cerebral ischemia can also be mediated via adoptive transfer of splenocytes from MBP-tolerized to naïve animals [186].

Employing tolerization to E-selectin, which is only expressed the luminal aspect of activated endothelium, tolerized animals were again protected against focal cerebral ischemia and this protection was also cell-mediated and transferable to naïve animals via splenocytes from tolerized animals [100]. Mechanistically, rodents tolerized to E-selectin had evidence of antigen-specific T-cell mediated Th1 type Delayed Type Hypersensitivity (DTH) reactions suggesting that antigen-specific, T-cell mediated suppression of pathogenic Th1 responses may be responsible, in part, for the cytoprotection observed in tolerized animals [100].

Collectively, these studies suggest that mucosally-induced tolerance to antigens expressed on activated vascular endothelium, such as E-selectin, lead to the development of antigen-specific T-cell mediated cytoprotection via secretion of immunomodulatory cytokines. It has been proposed that while the T-cell-mediated response to tolerization is antigen-specific, the secretion of immunomodulatory cytokines is not, and leads to neuroprotective by-stander immunosuppression [187].

Humoral immunity; B-cell mediated:

B lymphocytes play a role in the immune response to stroke as evidenced by the presence of antibodies to CNS antigens in the peripheral circulation of patients with acute cerebral ischemia [188]. In addition, transfer of regulatory B-cells into the brain is protective against experimental stroke, in the acute phase, possibly via decreases in inflammatory cytokine production by peripheral T cells. [189].

Further evidence supporting a role of B cells in the immune response to stroke is the presence of splenic atrophy in experimental stroke and significant decreases in circulating and splenic B-cell populations [190]. In contrast, splenectomy [191] or pretreatment with inflammatory modulators, such as statins, that decrease splenic atrophy [192], are protective against experimental stroke. It remains to be seen whether these findings can be confirmed in human stroke.

Post-stroke autoimmunity and immunodepression:

It is now fairly well established that stroke patients have immune suppression commonly referred to as post-stroke immunodepression which leads to an increased incidence of infections such as pneumonia and urinary tract infections (UTI) in the post-stroke period.

Mechanisms such as autoimmunity to CNS antigens and beta adrenergic over activation have been advanced to account for the immune dysfunction that occurs in acute stroke patients. Studies have shown that up to 30% of patients with acute stroke have infections such as pneumonia and UTI [193]. It is not known however, how many of these infections can be attributed to post-stroke immunodepression.

Autoimmunity:

In this model, post-stroke immunodepression is thought to be an adaptive response to help prevent unwanted autoimmunity against CNS antigens. In support of this hypothesis are studies that have shown a correlation between the amount of infarcted brain tissue and the

concentrations of CNS antigens such as MBP, creatine kinase, neuron-specific enolase and s100 in the peripheral circulation of stroke patients [194]. Furthermore, a robust TH1-type response against some of these CNS antigens has been associated with poor 3-month functional outcome following acute stroke. Specifically, in a study of 114 acute stroke patients testing the hypothesis of an association between poor outcome post-stroke and a Th1 response against brain antigens, for each one point increase in the NIHSS score (a measure of stroke severity), the odds of developing a Th1 response to a CNS antigen such as MBP increased nearly 10% [195]. It remains to be determined whether stroke-related autoimmunity to brain antigens is as a consequence of or causal for post-stroke immunodepression.

Adrenergic over activation and adrenergic-responsive immune cells:

Another mechanism advanced for post-stroke immunodepression is over activation of the adrenergic system leading to the release of catecholamines and corticosteroids. This model is supported by studies showing increased serum cortisol and catecholamine levels in patients with acute stroke with elevated cortisol levels being an independent predictor of mortality in these patients [196]. Metabolites of catecholamines such as metanephrine have also been associated with increased 3-month mortality in acute stroke patients in other studies [197]. In addition, in experimental stroke models, beta blockade reduces propagation of intranasal colonization, and aspiration with *Strep Pneumoniae*, to pneumonia further implicating adrenergic over activation in the pathogenesis of stroke-induced immunodepression [198]. Furthermore, in a retrospective study, use of beta blockers was associated with decreased early mortality in patients with acute stroke [199].

More recent studies suggest that previously unknown neuroimmune mechanisms may be responsible, in part, for post-stroke immunodepression. Along these lines, immune cells such as T cells have been found to secrete catecholamines and to express β -adrenergic receptors. In addition, adrenergic nerve terminals in peripheral organs are thought to mediate the adrenergic effects proposed to be responsible for post-stroke immunodepression [200]. In support of this proposal are findings that depletion of noradrenergic terminals in the liver, or β -adrenergic blockade of these receptors, with a beta blocker, alters cytokine expression by a group of immune cells; hepatic invariant Natural Killer (iNKT) cells, activated by stroke, resulting in decreased immunosuppression, bacterial infections and decreased mortality in an experimental stroke model [200].

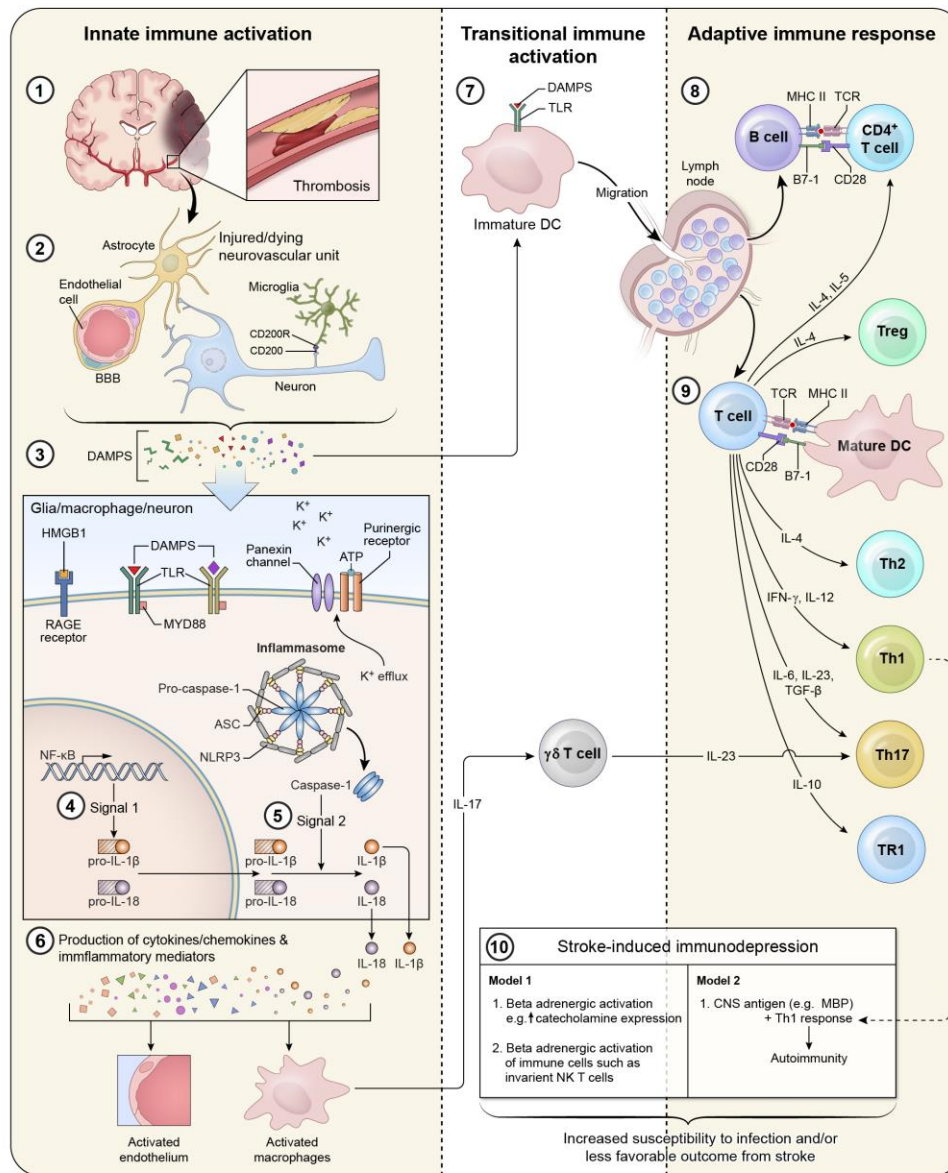


Figure 1. The immune response to focal cerebral ischemia: 1) Focal cerebral ischemia secondary to thrombotic or embolic event. 2) Injury to neurovascular unit resulting in either death or injury to endothelial cells, neurons, microglia etc. 3) Release of DAMPs from injured and dead neurovascular unit components resulting in innate immune activation via TLRs, RAGE and purinergic receptors on microglia and other APCs. 4) Innate immune activation leading to inflammasome activation; Signal 1= expression of pro-IL-1 β and pro-IL-18. 5) Inflammasome activation; Signal 2= activation of caspase 1 and production of mature IL-1 β and IL-18. 6) Production of other cytokine via NF- κ B activation leading to endothelial cell and macrophage activation as well as activation of other transitional immune effector such as $\gamma\delta$ T cells. 7) Activation of other transitional immune effectors such as immature DC, via DAMPs, and surface TLRs and migration of these immature DCs to regional lymph nodes resulting in activation of the adaptive immune response. 8) Activation and differentiation of B cells. 9) Activation and differentiation of T cells. 10) Models of stroke-induced immunodepression via beta adrenergic or Th1-mediated mechanisms. BBB, Blood brain barrier; CD200R, CD200 receptor; DAMP, Danger associated molecular pattern; HMGB1, High mobility group box 1; RAGE, Receptor for advanced glycation end products; MyD88, Myeloid differentiation primary response gene 88; ASC, Apoptosis-associated speck-like protein containing a CARD (Caspase activation and recruitment domain); IL, Interleukin; DC, Dendritic cell; TCR, T cell receptor; MHC, Major histocompatibility complex; CD, Cell differentiation; Treg, T regulatory; Th, T helper; TGF, Transforming growth factor; TR1, Type 1 regulatory T cell; CNS, Central nervous system; NK, Natural killer; MBP, Myelin basic protein.

Prophylactic antibiotic treatment:

Clinical trials that have sought to utilize prophylactic antibiotic treatment to pre-empt the propensity towards post-stroke infections in stroke patients have shown mixed results. In the ESPIAS trial, prophylactic antibiotic treatment had no effect on infection rate or on acute stroke mortality [201]. In contrast, in the PANTHERIS trial; albeit with a different antibiotic, the infection rate was decreased in patients treated with prophylactic antibiotics, but the mortality rate was not significantly different compared to patients not on antibiotics [202]. The results of the ongoing randomized, controlled, phase 3 trial; Preventive Antibiotics in Stroke Study (PASS), which tests the hypothesis that preventative antibiotics improves functional outcome after stroke, will provide more information regarding the potential long-term benefits of preventive antibiotic use in stroke patients [203].

The mixed results from the antibiotic prophylaxis trials may be reflective of the fact that prophylactic antibiotic treatment does not counteract the endogenous mechanisms thought to be responsible for post-stroke immunodepression. Along these lines, a follow-up study to determine the cellular immunological mechanisms responsible for infection in the patients from the PANTHERIS trial, found that infection after stroke was associated with profound T-cell lymphocytopenia, reduced interferon gamma secretion, T cell deactivation and increased sympathetic activity as assessed by urinary norepinephrine levels [172]. However, the finding of T-cell deactivation in this population of stroke patients is in contradiction to the evidence of T-cell activation found in the serum of another group of acute stroke patients [22].

Conclusions

In summary, the current state of knowledge regarding activation of the immune response to focal cerebral ischemia is not complete, but continues to evolve. However, it is important that the level of understanding of the intricate regulation of this powerful endogenous immune response to cerebral ischemia continues to be advanced. Since available research tools and knowledge in biomedical research in general continues to advance rapidly, the gaps in our understanding of this powerful endogenous response to cerebral ischemia can only be expected to grow in the years to come. The current understanding of innate and adaptive immune activation in response to focal cerebral ischemia, as well as post-stroke immunodepression is summarized in Figure 1.

Some of the areas that will need particular focus include understanding the reasons for the differences in

activity of specific injury-induced cytokines and chemokines in the acute versus late phase of cerebral ischemia. Also, understanding the intermediate immunological mechanisms uniquely placed between the innate and adaptive immune responses is important for understanding the factors that control the switch from the innate to adaptive immune response following focal cerebral ischemia. It is also essential to explore and understand the differences between the innate immune response to transient versus permanent cerebral ischemia, as well as the differences between immune activation in response to cerebral ischemia in younger versus older patients. Likewise, it is important to understand the differences in the activation of the immune response to cerebral ischemia in patients with underlying chronic inflammatory conditions versus in patients without these underlying comorbidities. The molecular mechanisms that underlie immunodepression following cerebral ischemia also require continued investigation. Finally, the neuroimmune mechanisms that inform the effect of stroke on the peripheral immune response, and vice-versa, require ongoing research. These types of advances should lead to the development of novel therapeutic strategies aimed at specific time points following cerebral ischemia.

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